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Research paper

Genome-wide identification and profiling of microRNAs in *Paulownia tomentosa* cambial tissues in response to seasonal changes

Zongbo Qiu*, Yanyan He, Yimeng Zhang, Junli Guo, Liang Zhang

College of Life Science, Henan Normal University, Xinxiang 453007, PR China

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ABSTRACT

MicroRNAs (miRNAs), a group of endogenous small non-coding RNAs, have been shown to play essential roles in the regulation of gene expression at the post-transcriptional level. Although *Paulownia tomentosa* is an ecologically and economically important timber species due to its rapid growth, few efforts have focused on small RNAs (sRNAs) in the cambial tissues during winter and summer transition. In the present study, we identified 33 known miRNA families and 29 novel miRNAs which include 20 putative novel miRNAs* in *P. tomentosa* cambial tissues during winter and summer transition. Through differential expression analysis, we showed that 15 known miRNAs and 8 novel miRNAs were preferentially abundant in certain stage of cambial tissues. Based on the *P. tomentosa* mRNA transcriptome database, 1667 and 78 potential targets were predicted for 29 known and 20 novel miRNAs, respectively and the predicted targets are mostly transcription factors and functional genes. The targets of these miRNAs were enriched in "metabolic process" and "transcription regulation" by using Gene Ontology enrichment analysis. In addition, KEGG pathway analyses revealed the involvement of miRNAs in starch and sucrose metabolism and plant-pathogen interaction metabolism pathways. Noticeably, qRT-PCR expression analysis demonstrated that 9 miRNAs and their targets were existed a negative correlation in *P. tomentosa* cambial tissues. This study is the first to examine known and novel miRNAs and their potential targets in *P. tomentosa* cambial tissues during winter and summer transition and identify several candidate genes potentially regulating cambial phase transition, and thus provide a framework for further understanding of miRNAs functions in the regulation of cambial phase transition and wood formation in trees.

1. Introduction

Wood represents the abundant source for terrestrial biomass production and is one of the most important sinks for atmospheric carbon dioxide. Wood formation begins from the activity of vascular cambium and generates wood as the end product of secondary vascular system development (Mishima et al., 2014). Vascular cambium, an important lateral meristem, gives rise to secondary phloem to the outside and secondary xylem (wood) to the inside. Furthermore, the cambium of trees exhibits a seasonally cyclical pattern starting with activation of the vascular cambium at the beginning of the growing season and ending with cambium dormancy at the end of the season (Liu et al., 2014; Zhang et al., 2014). The transition from plant dormancy and active growth is a complex biological phenomenon that involves a considerable number of genes and many metabolic processes, as well as the interactions of various hormones (Qiu et al., 2013; Ding et al., 2014; Mishima et al., 2014). Multi-levels regulatory networks are involved in such developmental processes, in addition to transcriptional, epigenetic and environmental signals regulation.

MicroRNAs (miRNAs) are small 20–24 nucleotide (nt) long non-coding RNAs derived from hairpin-shaped precursors (pre-miRNA). They act as important regulators involved in various biological processes, such as phase transition, pathogen defense and adaptation to abiotic stress in woody plants by controlling gene expression (Qiu et al., 2015; C. Zhang et al., 2016). A recent study on miRNAs in *Paulownia fortune* indicated that 142 conserved and 38 novel miRNAs were predominantly expressed in the leaves (Niu et al., 2014). In diploid and autotetraploid *Paulownia tomentosa*, 37 conserved miRNA families and 14 novel miRNAs were identified by using high-throughput sequencing technology (Fan et al., 2014). Similarly, Zhai et al. (2016) discovered

Abbreviations: miRNAs, microRNAs; nt, nucleotide; scRNA, small cytoplasmic RNA; snRNA, small nuclear RNA; snoRNA, small nucleolar RNA; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MFE, minimum folding free energy; MFEI, minimal folding free energy index; bZIPs, basic-leucine zippers; SPLs, squamosa promoter-binding proteins; ARF, auxin response factor

* Corresponding author at: College of Life Science, Henan Normal University, 46 Jianshe Road, Xinxiang 453007, PR China.
E-mail address: qiuzongbo@htu.edu.cn (Z. Qiu).

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49 conserved miRNAs and 25 novel miRNAs in hybrid varieties *Paulownia* 'Yuza 1' using small RNA sequencing. Although significant progress has been made in detecting novel miRNAs in trees, there is little information on genome-wide characterization of miRNAs and their targets in the cambial tissues during winter-summer growth transition and very little is known about the regulation of miRNAs in this process.

Paulownia tomentosa is an indigenous fast-growing tree species with great ecological and economic value in China, where it has a very wide distribution and thus serves as a global resource for wood (Fan et al., 2014). Owing to the lack of *Paulownia* whole genomic sequences, we have little information of the regulation of cambium activity and phase change in *P. tomentosa* at the molecular level. Here, we employed high-throughput sequencing and bioinformatics analysis to comprehensively identify miRNAs and their targets in *P. tomentosa* cambial tissues during winter-summer growth transition. The findings from this study will not only contribute much to our understanding of miRNA-mediated regulation in the cambial tissues, but also lay a foundation for further investigations into the molecular mechanisms of phase change in tree.

2. Materials and methods

2.1. Plant materials

Tissue samples were harvested from the stems of *P. tomentosa.* These trees were approximately 20 years old, growing under good conditions in the Institute of Paulownia, Henan Agricultural University, China. The bark and phloem were peeled off and a thin layer of cambium with little of the surrounding phloem tissues was collected during the winter (20/December/2015), less-actively growing season and during the summer (12/July/2016), the actively growing season. The harvested cambial tissues were immediately frozen in liquid nitrogen and then stored at −80 °C. Six bulked samples (5 trees each) were made by grinding and mixing the sampled material. Three bulks represented summer trees (marked SC) and three represented winter trees (marked WC).

2.2. Anatomical observation of Paulownia vascular cambium

Blocks including secondary phloem, vascular cambium, and secondary xylem cut from the stems of *P. tomentosa* were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). After dehydration through an ethanol gradient (30, 50, 70, 80, 90, and 100%), the samples were embedded into Spurr's resin and sectioned using a Leica microtome. Semi-thin sections (5 μm) were stained with toluidine blue O (Sigma), and observed under a Zeiss Axioskop 2 Plus microscope equipped with a computer-assisted digital camera.

2.3. Total RNA isolation, small RNA library construction and sequencing

Total RNA was extracted from the cambial tissues using the method described by Qiu et al. (2015), followed by quantity and integrity assessment using Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). Small RNA libraries from SC and WC were constructed as depicted by Cakir et al. (2016) and sequenced using an Illumina HiSeq™ 2000 at Novogene Bioinformatics Technology Co. Ltd. in Beijing, China.

2.4. Computational analysis of sequencing data

The raw reads from high-throughput sequencing were first filtered to remove poor quality reads, poly A and adapter sequences using in-house program, ACGT101-miR v4.2 (LC Sciences, Houston, TX, USA). Subsequently, the clean reads were obtained. The length distribution of the clean reads was determined and the reads were mapped to the *P. tomentosa* mRNA transcriptome database (NCBI SRR5091910) by using Bowtie software (Langmead et al., 2009) with perfect match. Clean reads with a length of 18–30 nt were compared against GenBank (http://www.ncbi.nlm.nih.gov/), RepBase (http://www.girinst.org/) and Rfam (version 10.1) (http://rfam.sanger.ac.uk) databases to annotate RNA categories including tRNA, rRNA, small cytoplasmic RNA (scRNA), small nuclear RNA (snRNA), and small nucleolar RNA (snoRNA) from each database. To identify known miRNAs, the remaining sRNA sequences were matched to known miRNAs sequences from all plant species in miRBase 21.0 (http://www.mirbase.org) using a modified software, miRDeep2 (Friedlander et al., 2011) and srna-tools-cli with no more than two mismatch. The sequences, not being annotated to known miRNAs and other kinds of sRNAs, were used to predict novel miRNAs by the software miREvo (Wen et al., 2012) and miRDeep2 (Friedlander et al., 2011). To further obtain known and novel miRNA precursors, the unique sRNAs were aligned to the *P. tomentosa* mRNA transcriptome database (NCBI SRR5091910) using the software Bowtie with default parameters. These precursor sequences were retrieved and used for BLASTX analysis to remove the protein coding sequences. Then, prediction of the stem-loop structure of pre-miRNA was performed by use of the RNAfold web server (Zuker, 2003) (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) with the default parameters. DEGseq package (1.8.3) was used for differential expression analysis of miRNAs between two samples. The criteria of $|\log_2$ fold change$| \geq 2$ and $P \leq 0.01$ was set as the threshold for defining significantly different expression.

2.5. Target gene prediction and functional annotation

The potential targets of known and newly identified *P. tomentosa* miRNAs were predicted using psRNATarget program (http://plantgrn.noble.org/psRNATarget/) (version 12) by using the miRNA sequences as queries for a search against the *P. tomentosa* transcriptome (NCBI SRR5091910) and NCBI's EST and GSS sequences. The *Paulownia* transcriptome contains 104,432 unigene sequences with an average length of 662 bp. In addition, we used Gene Ontology (GO) enrichment analysis (http://www. geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (http://www.genome.jp/kegg/) to further probe the functions of these targets of the differentially expressed miRNAs.

2.6. Verification of miRNAs and their target genes by real-time quantitative RT-PCR

To validate the expression levels of miRNAs identified by deep sequencing, nine miRNAs and corresponding targets were selected randomly and determined by qRT-PCR on the Rotor-Gene 3000 real-time PCR detection system (Qiagen) using TransStart Green qPCR SuperMix UDG (Trans) according to the manufacturer's instructions. The primers of nine randomly selected miRNAs were designed according to the sequence of pre-miRNA, and all primers for the miRNAs and their targets are listed in Table S1. qRT-PCR was carried out according to the method described by Qiu et al. (2015) with three biological replicates and 18S rRNA was used as a reference gene. Relative expression levels were calculated by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and Duncan's multiple range test was performed to compare differences between winter and summer cambial tissues.

3. Results

3.1. Anatomical structure of Paulownia vascular cambium

The vascular cambium underwent seasonal cycles, termed the dormant and active cambium. As shown in Fig. 1, it is obvious that the cambial zone has 2–3 layers of cells containing a thick cell wall and was easily distinguished from mature xylem and secondary phloem in the samples collected in the middle of December (Fig. 1A, B). In samples collected in the middle of July, the cambial zone has 7–8 layers of cells with a thin cell wall, which enter the vigorous active stage (Fig. 1C, D). These results provided a cytological foundation for further studies on

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